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(54) **Monoclonal antibodies to preS2 and preS1 polypeptides of the hepatitis B viral envelope.**

(57) Murine monoclonal antibodies are provided which bind specifically to the PreS1 and PreS2 polypeptides of the hepatitis B virus surface antigen (HBsAg) complex and which are useful to assay for PreS1 or PreS2 in samples of physiological material. The antibodies can also be used to subtype HBV, to raise the immunogenicity of blood plasma against HBV, to measure anti-PreS1 and anti-PreS2 antibodies, and to identify and characterize antigenic epitopes of PreS1 and PreS2 which may be useful in HBV subunit vaccines.

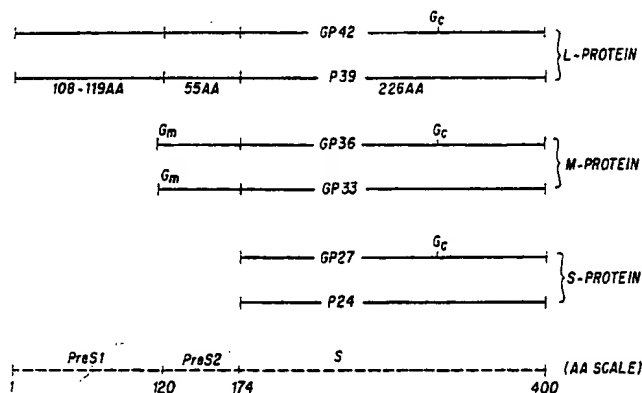


FIG. 1

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MONOCLONAL ANTIBODIES TO PRES2 AND PRES1 POLYPEPTIDES OF THE HEPATITIS B VIRAL ENVELOPE

Cross-Reference to Related Application

This application is a continuation-in-part of U.S. Patent Application Serial No. 07/332,014, filed March 31, 1989.

Background of the Invention

Hepatitis B virus (HBV) infections represent a major public health problem throughout the world. Vaccination is the most efficient way to prevent acute hepatitis B infection and its chronic sequelae, e.g., liver cirrhosis and hepatoma. A hepatitis B vaccine used with good success consists of viral surface antigen (HBsAg) which is isolated from the blood of chronic virus carriers as noninfectious 22 nm particles. The 22 nm particles primarily contain a major protein of 24,000 d (p24) and its glycosylated form, gp27. Recently introduced vaccines comprise p24 which is synthesized by yeast cells that have been transfected with HBV gene S, which codes for p24. The safety and efficacy of these vaccines have been established but their immunogenicity is insufficient in a certain proportion of recipients. Thus, a need exists for more immunogenic HBV vaccines, the identification of which will require a more advanced knowledge of the location, structure, and immunogenicity of antigenic sites on HBV proteins.

The protein composition of the HBV envelope has been determined and mapped within the viral genome. Figure 1 summarizes a model proposed by N. Herrmann et al., *J. Virol.*, 52, 396 (1984). In this model, the three HBV envelope proteins and their glycosylated forms are derived from one continuous open reading frame (ORF), which is divided into the S, PreS2, and PreS1 regions. The S gene starts at the third or fourth start codon of this ORF, depending on the viral subtype, and codes for the major HBV envelope protein, the S protein, which includes p24 and its glycosylated form gp27. The PreS region contains two additional initiation sites in the same reading frame with the S gene allowing the expression of two larger hepatitis B surface antigen molecules: M protein (consisting of the 55 amino acids of PreS2 + S) and L protein (consisting of the 108 and 119 amino acids of the PreS1 + PreS2 + S protein). M protein consists of singly or doubly glycosylated forms, gp33 and gp36, respectively. L protein consists of an unglycosylated and a singly glycosylated form, p39 and gp41, respectively.

In the blood of HBV infected persons, only a very small portion of the total hepatitis B surface antigen exists in complete virions (the Dane particles). Two other morphological forms, 22 nm spherical particles and filaments 22 nm in diameter and of variable length, lack capsid or DNA and are produced in an excess which may be as high as 1×10^6 :1 over the 42 nm "Dane" particles. The protein composition of these morphological forms differs considerably. Up to 20% of total envelope protein in HBV Dane particles is L protein compared to only about 1% of total protein in the 22 nm spherical particles.

Overproduction of L protein in transfected cells leads to inhibition of secretion of L protein particles from these cells. It has been hypothesized that the L protein may play an essential role in the secretion and morphogenesis of complete virions.

The PreS1 regions of L protein may contain a specific receptor for the human hepatocytes as indicated by its ability to bind to Hep G2 cells (A. R. Neurath et al., *Cell*, 46, 429 (1986)). Specific binding of the PreS2 region to polymerized human serum albumin has also been proposed as a possible mechanism for association of HBV with the hepatocyte plasma membrane (M. Imai et al., *Gastroenterology*, 76, 242 (1979)).

Regardless of the role of PreS-containing proteins in viral structure or function, PreS1 and PreS2 proteins have been shown to be highly immunogenic in mice and their combination with S protein may potentiate the immune response to the S protein (D. R. Milich et al., *Science*, 228, 1195 (1985)). A synthetic peptide vaccine containing only a portion of the PreS2 region elicited an immune response to PreS2 and protected the inoculated chimpanzees from HBV infections after challenge with virus (A. Neurath et al., *Vaccine*, 4, 35 (1986)). The available hepatitis B vaccines differ significantly in their PreS composition, and the relative immunogenicity of PreS-containing vaccines in humans remains to be elucidated.

Ausria[®]II, Auszyme[®]II and monoclonal Auszyme[®] are commercially available assays for the detection of hepatitis B surface antigen (HBsAg) (Abbott Laboratories, N. Chicago, IL). Monoclonal Auszyme uses only

monoclonal antibodies to protein S. These HBsAg assays do not allow discrimination between L, M, and S proteins.

A commercially-available direct solid-phase radio-immunoassay (RIA) for anti-HBs detection and quantitation has been available since 1975 (AUSAB, Abbott Laboratories) (I. K. Mushahwar et al., *J. Med. Virol.*, 2, 77 (1978)). In this assay, the serum or plasma specimen is incubated with a solid-phase (polystyrene beads that has been coated with human plasma-derived hepatitis B surface antigen (hpHBsAg). If antibodies to HBsAg (anti-HBs) are present in the serum, they will bind to the solid-phase antigen. In a second step, radiolabelled HBsAg (^{125}I -HBsAg) is added and reacts with the immobilized antibody. The amount of radiolabel bound to the solid phase, within limits, is in direct proportion to the amount of anti-HBs in the original specimen. An enzyme-linked immunoassay (EIA) version of this test (AUSAB EIA) has been available since 1983 and uses a biotinylated HBsAg-horseradish peroxidase (HRP) conjugated avidin mixture as a probe "detecting" reagent (I. Mushahwar, *J. Virol. Methods*, 16, 1 (1987)). In an improved version of this test, a biotin/antibiotin detection system has been substituted for the biotin/avidin detection reagent (I. Mushahwar et al., *J. Virol. Methods*, 16, 45 (1978)).

However, these assays detect primarily anti-S antibodies. They may not detect anti-PreS1 and anti-PreS2 antibodies, and certainly do not discriminate between samples which contain predominantly anti-PreS1, anti-PreS2 or anti-S antibodies. A need exists for assays which can detect both PreS1 and PreS2 proteins and antibodies against them, since several investigators have reported that antibodies to PreS1 and PreS2 are often the earliest antibody response to HBV infection in humans, may be linked to viral clearance, and may act as a neutralizing Ab. For example, see Klinkert et al., *J. Virol.*, 58, 522 (1986). Hess et al., *Liver*, 7, 245 (1978), have shown a relationship between HBV induced cytopathology and the presence of PreS1 staining in cells from liver biopsy, but the potential pathogenic role of PreS1 expression is unknown. Alberti et al., *Hepatology*, 7, 207 (1978), found that the presence of antibodies against PreS2 correlated with evidence of termination or viral replication and onset of convalescence. Therefore, the administration of PreS1 antibodies and/or PreS2 antibodies to exposed individuals or to HBV patients may be an important adjunct to current therapeutic regimens using hyperimmune globulin (HBIG). Since some HBIG preparations may comprise antibodies to PreS1 and/or PreS2, a need exists for an assay which can determine the amounts of these antibodies.

HBsAg is also antigenically complex, containing group-specific "a" determinants, defined as being present on all HBsAg particles, and two sets of what are normally mutually exclusive subtype-specific determinants, d/y and w/r. This gives four major subtypes of HBsAg which are designated adw, adr, ayw, and ayr. Additional determinants have also been described resulting in universal recognition of ten different HBsAg subtypes. For example, two of these subtypes are designated adyw and adywr, and represent instances where both "d" and "y" or "w" and "r" are found to exist in the same sample. The amino acid sequences for five distinct HBV subtypes of PreS2 are shown by A. R. Neurath et al., in *Science*, 224, 392 (1984)).

Mixed subtypes such as these presumably result from infection of cells with two or more viruses of separate genotype. The resulting mixed phenotype then occurs either as a mixture of particles with different subtypes, or as particles which contain both subtypes. In the case where both subtype determinants occur on each particle, the mixed subtype particle is believed to result simply from phenotypic mixing, that is, from the intermixing of the two different subtypes of p25 and gp30 protein coded for by the two different viral S genes, upon assembly of the viral envelope. Particles composed of both forms of this protein would thus have both sets of subtype determinants. Upon viral transmission, particles with separate subtypes would be produced. See D. A. Paul et al., *J. Virol. Methods*, 13, 43 (1986). This complexity has led to a continuing need for methods to determine the HBsAg subtype of infected individuals. Tracking HBs subtypes can help to monitor the epidemiology of HBV infection, and elucidate the origin of mixed HBV subtypes.

Summary of the Invention

The present invention provides two panels of monoclonal antibodies (mAbs) which define at least three distinct epitopes of the PreS1 protein and four distinct epitopes within the PreS2 protein of the HBV envelope. The isotypes and binding properties of these six "groups" of mAbs is described in detail hereinbelow. The epitope specificity for four of the groups is summarized on Table 1, below:

Table 1.

Monoclonal Antibodies to PreS1 and PreS2 Proteins				
PreS1	Epitope Includes: ^a	L Binding	M Binding	S Binding
Group 1	~ 27-35 72-102	+	-	-
Group 2		+	-	-
PreS2				
Group 1	120-145	+	+	-
Group 2	123 (+ glycan)	-	+	-

^a Amino acid position downstream from PreS1 methionine according to Figure 1 of A. R. Neurath et al., Cell, 46, 429 (1986).

These epitopes are specific to PreS1 or PreS2, e.g., they are retained after treatment of HBsAg-containing samples, such as Dane particles, which destroys essentially all of the antigenicity of the S-protein. Some of the mAbs from each group exhibit "high affinity" binding ($K = 10^9$ - 10^{12}) to their epitopes. These mAbs represent preferred embodiments of this aspect of the invention.

As defined herein, an epitope comprising a region of the PreS1 or PreS2 protein is contained at least in part by the amino acid sequence of the region as set forth in Table 1, and may also include amino acid sequences flanking or adjacent to said region.

Immunoassays

1. Antigen Assays

The present mAbs can be used to develop specific and sensitive immunoassays to detect HBV and HBsAg in physiological material such as human blood sera or plasma. If an HBV variant was so different from known variants that anti-S based detection systems, such as the Monoclonal Auszyme[®] assay, were unreactive, then the present anti-PreS based antigen detection assays could be used to screen the suspect specimens.

One assay which has been investigated using the present mAbs is a sandwich type radioimmunoassay (RIA) or enzyme-linked immunoassay (EIA) in which a liquid test sample comprising PreS1 or PreS2 protein, e.g., as the L and/or M protein, is reacted with a solid phase comprising a "capture antibody" for these proteins, which can be monoclonal or polyclonal. The capture antibody (Ab) can be one which binds to the S protein region, or one of the present mAbs, which binds specifically to an epitope in the PreS1 or PreS2 region. The captured PreS1 and/or PreS2 containing proteins are then detected and measured by reacting them with an anti-PreS1 or anti-PreS2 mAb of the present invention which has an attached detectable label, such as a radioisotope or an enzyme. Alternatively, the "detection antibody" can comprise a binding site for a detectable label, and the detectable label is added to the bound detection mAb in a separate step. The binding site for the detectable label can be an epitope which is bound by a third labelled antibody against the detection Ab. For example, enzyme-labelled anti-mouse antibody can be used to detect a bound murine mAb, such as those of the present invention. The amount of label bound to the captured PreS1- or PreS2-containing protein is then quantified, e.g., in counts per minute for a radiolabel or in absorbance (A) units for the color reaction catalyzed by an enzyme label, and correlated with standard PreS1- and PreS2-containing samples to yield a measure of the amount of PreS1- or PreS2-containing protein present in the test sample.

For example, the PreS1 antigen assays which have been developed using the present mAbs are summarized on Table 2, below.

Table 2

	Capture Ab on Solid Phase	Detection mAb
5	a) Group 1 PreS1	Group 2 PreS1
	b) Polyclonal Auszyme ^R (guinea pig; hyperimmune serum)	Group 1 PreS1
	c) Group 1 PreS1	Group 1 PreS1
	d) Group 2 PreS1	Group-2 PreS1
	e) Polyclonal Auszyme ^R	Group 1 + Group 2 PreS1
10	f) Monoclonal Auszyme ^R (mAb against S protein)	Group 1 and/or Group 2 PreS1

PreS2 antigen assays have also been developed using Group 1, 2, and/or 3 mAb PreS2 in combination or alone with polyclonal or monoclonal Auszyme^R beads or with PreS2 mAb bound to the solid phase.

Useful solid phases include plastic beads, such as the polystyrene beads used in the Auszyme^R assay; plastic microtiter plate wells and other plastic cell culture substrates, such as hollow fibers and sheets; woven or nonwoven fibrous solid phases such as those formed from paper, felt, or synthetic fibers, latex microparticles, porous ceramic beads such as silica, alumina or ZrO₂ beads and the like. The capture antibodies can be physically sorbed onto or into these substrates, or may be attached by covalent bonding methods known to the art.

The detection mAbs may be labelled by any of several techniques known to the art. A wide range of labelling techniques are disclosed in Feteanu et al., "Labeled Antibodies in Biology and Medicine", pages 214-309 (McGraw-Hill Int. Book Co., New York (1978)). The introduction of various metal radioisotopes may be accomplished according to the procedures of D. J. Hantowich et al., *Science*, 220, 613 (1983); Wagner et al., *J. Nucl. Med.*, 20, 428 (1979); Sundberg et al., *J. Med. Chem.*, 17 1304 (1974); and Saha et al., *J. Nucl. Med.*, 6, 542 (1976).

Among the radioisotopes used, x-ray-emitters, gamma-emitters, positron-emitters, and fluorescence-emitters are suitable for antibody detection. Preferred radioisotopes for labeling antibodies include Iodine 125, 131, or 123, Indium 111, Ruthenium 97, Copper 67, and Technetium 99. The halogens can be used more or less interchangeably.

One preferred labeling technique involves labeling with either Iodine-131 (I-131) or Iodine-125 (I-125) using an oxidative procedure wherein a mixture of radioactive potassium or sodium iodide and the antibody is treated with chloramine-T (N-chloro-p-toluene sulfonamide sodium salt) e.g., as reported by Greenwood et al., in *Biochem. J.*, 89, 114 (1963) and modified by McConahey et al., in *Int. Arch. Allergy Appl. Immunol.*, 29, 185 (1969).

In general, it is desirable to introduce as high a proportion of label as possible into the antibody molecule without destroying its immunospecificity. For each labelled antibody, the extent of the reduction in labeled antibody-antigen binding can be established by standard competitive cell binding assays. Preferably, at least about 30% of the initial binding activity of the antibody is preserved when 125-I or 131-I is used as the labels.

The present detection mAbs can be enzyme-labelled by direct covalent attachment of the enzyme or by use of a linkage such as the reaction between a biotinylated enzyme and an avidinated enzyme (see published European Patent Application No. 291,180, November 11, 1988). Useful enzyme labels include horseradish peroxidase (HRP), alkaline phosphatase, beta-galactosidase and glucose oxidase.

Because the present mAbs do not prevent the binding of target (M or L) protein to the solid phase, the present assays can also be carried in one step. In the one-step assay, the sample, the solid phase comprising the capture antibody and the detection mAb can be mixed together in the appropriate liquid phase. The amount of label bound to the solid phase is then detected as described hereinabove.

More specifically, one preferred two-step assay employs Auszyme^RII beads as the capture Ab and an HRP-conjugated or ¹²⁵I-PreS2 mAb of Group 1 or a Group 1/Group 2 mixture as the detection antibody. The sensitivity of this PreS2 assay was very high; about 1 ng PreS2 protein/ml total hpHBsAg or about 0.15 ng PreS2/ml of M protein can be detected. The assay was also specific for PreS2 to the extent that PreS2 protein can be detected in the presence of an 80,000-fold excess of experimentally-added S protein.

PreS1-containing L protein can be detected at ≤ 1 ng/ml sample without significant interference by added S protein, using capture Ab including a) monoclonal Auszyme^R beads, or b) Auszyme^RII beads, and a detection mAb comprising (a) HRP-labelled Group 1 PreS1 mAb; b) HRP or ¹²⁵I-labelled PreS1 Group 3 mAb, or c) ¹²⁵I-labelled Group 1 and Group 2 PreS1 mAb as the detection antibody. Use of a PreS1 mAb-coated bead eliminates any interference with the assay by added S protein. The appearance and/or

disappearance of PreS antigens in the blood of infected individuals has been reported to relate to the course of the disease and its prognosis. these assays may find clinical utility in the management of HBV. For example, assays which indicate only the presence or absence of these protein "markers," are not as useful as the present quantitative assays.

2. Antibody Assays

The present mAbs can be used as detection antibodies in competitive immunoassays which detect human antibody response to PreS1 and PreS2 regions on HBV. The preferred capture antigen is human plasma derived surface antigen (comprising S + M + L proteins) which is treated with a reducing agent (to cleave disulfide bonds) and iodoacetamide (to prevent reformation of disulfide bonds) to denature the S region. The denatured capture antigen is immobilized by adsorption onto a solid phase. The immobilized capture antigen is then combined with (a) a preselected amount of human blood serum or plasma which comprises endogenous HBV antigen (due to HBV infection) and (b) a preselected amount of a labelled PreS1 or PreS2-mAb of the invention. The solid phase is washed to free it of unbound material and the amount of the bound label is measured. The amount of bound, labelled mAb is inversely proportional to the amount of bound anti-HBV antibody. This assay provides a method to measure the concentration of anti-HBV antibody during the course of the disease, or to monitor antibody production in HBV vaccine recipients. This assay can also be used to measure endogenous or exogenous anti-PreS1 and anti-PreS2 antibodies in HBIG formulations.

Other antigens useful as capture antigens in a competitive sandwich assay include a mammalian polyclonal antibody which binds to non-PreS epitopes on a fusion protein comprising PreS protein. Such non-PreS epitopes are found, for example, on CKS (3'-CMP-KDO synthetase). The polyclonal antibody to CKS is immobilized and mixed with seropositive serum or plasma and preselected amounts of (a) a fusion protein containing at least a part of the CKS enzyme and a PreS antigen and (b) a labelled PreS mAb. The immobilized antibody binds to the CKS region of the recombinant antigen fusion protein comprising part of the CKS enzyme and PreS protein.

The PreS region of the fusion protein provides a binding site which is competitively bound by endogenous anti-PreS antibody and by the added labelled PreS mAb of the invention. Thus, the strength of the signal due to the bound label which is detected is inversely proportional to the amount of the anti-PreS antibody in the sample. As in the case of the PreS1 and PreS2 assays described hereinabove, the serum and detection mAb can be added sequentially to the capture antigen or can be mixed with the capture antigen in one step.

Subtyping HBsAg

The present invention also provides a method to determine the specific subtype of HBV present in a physiological fluid such as human blood serum or plasma. To develop this method, samples containing HBV of the known subtypes are first contacted with samples of a monoclonal or polyclonal anti-S-antibody which has been bound to a solid support. Binding of S-containing proteins also captures PreS1 and PreS2 proteins, such as those in the L and M protein. The members of the subtype panel of bound proteins are then divided into two portions and reacted sequentially with pairs of PreS1 or PreS2 mAbs from the same or different groups, wherein the mAbs comprise a detectable label. Optionally, a plurality of dilutions of the sample are reacted with each mAb. The amount of the bound label is then quantified.

For selected pairs of mAbs, the relative amount of bound label in a series of samples of known subtype gives rise to patterns which are unique to a particular subtype. Since the ay and ad subtypes can be readily identified using a pair of known anti-S mAbs, "scanning" a known ay or ad HBsAg sample with pairs of the PreS1 or PreS2 mAbs can effectively complete the typing. For example, the ratio of label, e.g., in counts per minute, for two PreS2, Group 1 mAbs, 50-80-194 and 116-83-406 obtained following their reaction with a HBsAg of ayw1 subtype, is more than 50 times that measured for any other HBV subtype. Ratio increases or decreases above average (taken as 1) of about 5-7 can be used to discriminate among subtypes.

This represents the first use of mAbs which specifically bind the PreS1 or PreS2 proteins in HBV subtyping. The observed patterns of reactivity of the present mAbs with a larger number of individual plasma or sera samples can be used to develop an algorithm for their use as subtyping reagents as reported by Courouce et al., Develop. Biol. Standard, 54, 527 (1982) and J. R. Wands et al., PNAS USA, 81,

2737 (1984), in the case of S-specific mAbs. The mAbs of the present invention may be useful to more fully analyze the heterogeneity in the antigenic properties of HBV strains during horizontal transmission and in various population groups that would be missed with anti-S mAb-based subtyping methods. This is particularly true in view of the relatively small antigenic region of the S protein.

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Vaccines and Monoclonal Antibody Therapy

At least some of the epitopes recognized by the present mAbs are immunodominant in the sense that endogenous antibody which competes with murine mAbs against these epitopes can be detected in seropositive individuals. These epitopes include the PreS2, Group 2 Asn 123 epitope listed on Tables 1 and 4. Therefore, the present mAbs can be used to detect, characterize, and isolate epitopal sites on PreS1 and PreS2 that may be useful components of subunit HBV vaccines. These vaccines may also be useful to increase the titer of PreS1- and/or PreS2-antibodies in humans, so as to provide a source of HBIG which is enriched in these antibodies.

The present invention is also directed to a method for raising plasma immunogenicity to HBV by administering a pharmaceutical unit dosage form comprising one or more of the present antibodies to a patient who may be or has been exposed to HBV. Therefore, it is believed that the antibodies of the present invention will have therapeutic potential, either as a prophylactic agent to improve the prognosis of infected and/or diseased patients by retarding the clinical progression of the disease, or possibly, as a curative agent which can act to eliminate the virulence of the virus. The present antibodies might also represent a valuable adjunct to hepatitis B immune globulin (HBIG). HBIG therapy with supplemental PreS1 and/or PreS2 mAbs may be useful to prevent liver reinfection following liver transplant in HBV-infected individuals.

The isolated mAbs preferably are diluted with a pharmaceutically-acceptable liquid carrier, such as an aqueous IV fluid, prior to being assayed for bioactivity or administered as a unit dosage form *in vivo*. See Remington's Pharmaceutical Sciences, A. Osol, ed., Mack Pub. Co., Easton, PA (16th ed. 1980) at pages 1488-1496, the disclosure of which is incorporated by reference herein. The resultant solution is sterilized, e.g., by filtration. Preservatives commonly employed with IgG preparations, such as maltose, glycine or thimerosal, may be added in pharmaceutically-acceptable amounts.

The resulting solutions are preferably administered parenterally, e.g., by intravenous infusion or injection. The amount of mAb composition administered will vary widely, and will depend on the physique and physical condition of the HBV-infected patient. Such factors are necessarily empirical, and can be determined by the clinician, employing known HBV staging criteria. In some clinical situations, it may be necessary to administer a plurality of doses of the mAb composition, in order to neutralize the infectivity of viral particles as they are released from infected target cells.

Brief Description of the Drawing

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Figure 1 is a schematic depiction of the protein composition of the HBV envelope.

Detailed Description of the Invention

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The invention will be further described by reference to the following detailed examples wherein Protein A-Sepharose CL-4B, CNBr activated Sepharose CL-4B, Sepharose S-300 and all other chromatographic gels were from Pharmacia (Piscataway, NJ). Electrophoresis and Western blot reagents were obtained from Bio-Rad, Richmond, CA. Horseradish peroxidase (HRP) was from Toyoba, New York, NY. Na¹²⁵I was obtained from Amersham, Arlington Heights, IL. The nine-member HBsAg subtype panel was from A-M Courouce (from the International Workshop of HBsAg Subtypes, Paris, April, 1975). Recombinant PreS1 (rPreS1) and recombinant PreS2 (rPreS2) were gifts from G. Okasinski (Abbott Laboratories, N. Chicago, IL). rPreS1 is a fusion protein which has a amino terminal portion of CMP-KDO synthetase (CKS) and a carboxy terminus containing PreS1 residue 12-120. The rPreS2 was a fusion protein with amino terminal portion of CKS and a carboxy terminus comprising PreS2 residues 123-175. Both rPreS1 and rPreS2 were HBV subtype adw2. Monoclonal antibodies (mAb) 18/1 and Q 19/10 were gifts from W. Gerlich (see K. H. Herrmann et al., J. Virol., 52, 396 (1984)).

Serum samples were tested with commercial RIA-EIA reagents supplied by Abbott Laboratories:

Monoclonal Auszyme^R and Ausria^{RII} for HBsAg; Corab^R for anti-HBc, Abbott HBe^R for HBeAg and anti-HBe and Abbott Genostics^R to detect HBV DNA.

To test for HBcAg in Dane preparations, samples (0.2 ml) were incubated with Monoclonal Auszyme beads for 2 hrs at 40° C. Beads were washed with water, then incubated with 200 µl of 1% Tween 20 in Tris buffered saline for 30 minutes at 40° C. Beads were washed and incubated with solution containing Monoclonal anti-HBc conjugated to HRP. Beads were washed and incubated with the substrate solution described above.

Example 1 - Monoclonal Antibodies to PreS1 and PreS2

A. Dane Particle Purification

Human plasma with a high HBsAg titer was clarified by centrifugation at 2000 rpm for 20 minutes. The supernatant was centrifuged for 8 hr at 27,000 rpm in a SW28 rotor. The resulting pellet was resuspended in 0.01 M Tris, 1 mM EDTA, pH 7.6 (Buffer A) and this resuspension mixture was layered on 10% sucrose in buffer A which had been overlaid on a 2 ml 62% sucrose/Buffer A pad. After centrifugation for 8 hrs at 27,000 rpm in a SW28 rotor, fractions were collected and assayed for HBcAg. Peak fractions of HBcAg were pooled, diluted 1:4 in Buffer A, overlaid on a continuous 15-62% sucrose gradient, and spun for 24 hrs at 27,000 rpm. Fractions were collected and assayed for HBsAg, PreS2 Ag, PreS1 Ag, and HBcAg.

B. Immunizations

Female 8-week old BALB/c mice were immunized with 10 µg of purified Dane particles emulsified in MPL + TDM adjuvant (#R-700, Ribi Immunochem Research, Inc.) three times at three-week intervals. Serum samples were drawn two weeks after the last boost and titers were evaluated by enzyme-linked immunoassay (EIA) and Western blotting.

C. Immunoassay for the Detection of Anti-Dane Antibody

Dane particles denatured in 5% beta-mercaptoethanol (BME), 1% SDS and boiled for 3 minutes were diluted to 2 µg/ml in PBS and coated onto microplate wells during an overnight incubation at room temperature. Wells were washed with distilled water and blocked for 30 minutes with 200 µl of 3% BSA in PBS. Plates were washed and allowed to air dry. In the assay, the specimen was incubated 2 hr at room temperatures in the wells, incubation wells were washed, and HRP conjugated goat anti-mouse-Ig (#14-18-09, Kirkegaard & Perry Laboratories, Inc.) was added and incubated 1 hr. After the final wash, 100 µl of an OPD substrate solution (Abbott Laboratories) was added to each well. The color reaction was stopped by addition of 1 N sulfuric acid.

D. Fusion Procedure

Responding mice were rested 2-4 months, then given a pre-fusion i.v. boost of 10 µg Dane particles in phosphate buffered saline (PBS). Three days later, the splenocytes were fused 1:1 with the SP2/0 myeloma line using standard protocols with slight modifications (G. Kohler et al., Nature, 256, 495 (1975)). The fusion pellet was briskly dispersed with 1 ml 50% PEG (ATCC NW 1450) for 1 minute, centrifuged in 20 ml media, and cells were resuspended in HAT selective IMDM to be plated at 1.5×10^5 lymphocytes per well in 96-well tissue culture plates (#167008, NUNC). To promote hybrid survival, STM mitogen (#R-510, Ribi) was added into the initial plating media at a 1 to 200 dilution. The mitogen was not used in subsequent media changes and feedings.

E. Establishment of Clones

Hybrid supernatants which were found positive on the anti-Dane screening EIA were evaluated by Western blotting to identify specific banding patterns. EIA and Western blot positive hybrids were cloned by limiting dilution. The clones selected for evaluation were derived from plates with approximately 10% growth per plate. Established clones were assayed as described above, grown up in T flasks and 1×10^7 cells/mouse were injected into pristane-primed BALB/c mice (Dominion Labs) to generate mAb containing ascites.

F. Isotype Determination

MAB isotype was determined with the SBA Clonotyping System III kit (#5030, Southern Biotechnology Associates, Inc.) with slight modifications. EIA 96-well microplates were coated overnight at room temperature with 100 μ l/well of a 1:1000 dilution of goat anti-mouse IgG + M (H+L) (Kirkegaard and Perry Laboratories, Inc.) as described for Dane particles above. Plates were washed and 50 μ l of clone supernatants were added to appropriate wells for a 2 hr incubation at room temperature. Plates are washed and 100 μ l/well of a 1:1000 dilution of the kit isotype specific conjugates were added to each sample for a 30-minute incubation. After the final wash, chromagen was added as described above.

G. Monoclonal Antibody Purification

Antibody from IgG cloned lines was purified from mouse ascites fluid using a Affi-Gel Protein A MAPS II kit (Bio-Rad). Following equilibration of the column with binding buffer, ascites was mixed in a 1:1 ratio with binding buffer, passed through a 0.2 μ m filter and loaded onto the column. After the sample was loaded onto the column, the 10 to 15 column volumes of binding buffer was passed through the column. IgG was then eluted with the supplied elution buffer, neutralized to pH 7.2, and dialyzed in PBS overnight at 4° C.

H. Synthetic Peptides

Synthetic peptides corresponding to portions of the translational products of the PreS2 gene downstream from Met120 and the PreS1 gene downstream from Gly13 were made using an ABI solid phase peptide synthesizer (Applied Biosystems). Two peptides were constructed, then purified by high pressure liquid chromatography to yield PreS2: MQWNSTAFHQTLQDPRVRGLYLPAGG (120-145) and PreS1: GTNLSVPNPLGFFPDHQLDPAFGANSNNPOWDFNPVKDD (13-51), wherein single letter abbreviations for the amino acid residues are A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; F, phenylalanine; M, methionine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; and V, valine.

I. Peptide Inhibition of Mab Binding to Dane Particles

The synthetic peptides coding for PreS1 residues 13-51 and PreS2 residues 120-145 were used in inhibition assays. Microtiter EIA plates were coated by incubation of a PBS solution containing 2 μ g/ml denatured Dane particles for 18 hrs at room temperature. Prior to the addition of the mAbs to the EIA plates, mAbs at 10 μ g/ml were preincubated with 10 μ g of either the 13-51 or 120-145 peptide for 30 minutes. Fifty μ l/well of sample was added to the plate, incubated for 20 minutes, and washed. A 1:1000 dilution of HRP conjugated goat anti-mouse Ig (KPL) was added at 50 μ l/well, incubated 30 minutes, washed, and color developed with o-phenylenediamine (OPD) substrate.

J. Monoclonal Antibody Affinity

The antibody affinity was determined by the protocol of V. van Heyningen, *Methods in Enzymology*, J. J. Langone et al., eds., Academia Press, Inc. (1986) at page 472f. Antibodies were ranked as low ($K = 10^6$ - 10^7), intermediate ($K = 10^7$ - 10^9) and high affinity ($K = 10^9$ - 10^{12}). The affinity of mAbs in purified or unpurified culture supernatant was determined as the dilution factor or concentrations giving 50% maximal binding of the monoclonal antibody to denatured Dane particles. HRP conjugated goat anti-mouse Ig was used to

detect bound antibody.

K. Polyacrylamide Gel Electrophoresis/Western Blots

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Analytical polyacrylamide gel electrophoresis (PAGE) was performed with a Bio-Rad slab gel apparatus with 12% running gel and 4% stacking gel. The gel and buffer formulations were those of U. K. Laemmli, Nature, 227, 680 (1970). Specimen was generally boiled for 5 minutes in a 1% SDS, 2.52% BME in Tris buffer. Western blotting was conducted essentially as described by H. Towbin et al., J. Immunol. Methods, 10 72, 313 (1984). After transfer of specimens onto nitrocellulose, nitrocellulose was soaked in blocking buffer (1% bovine hemoglobin, 0.1% v/v Tween 20 in PBS). Nitrocellulose was incubated with mAb to be tested at 0.5 to 1 μ g/ml in blocking buffer for 1-2 hrs, then washed with phosphate-buffered saline (PBS), and incubated for 1-2 hrs with HRP conjugated goat anti-mouse IgG or IgM (0.5-1.0 μ g/ml). Strips are washed, then developed with 4-chloro-1-naphthol/hydrogen peroxide substrate.

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L. Radioiodination and Enzyme Conjugation

MAB protein concentration was determined in mg/ml from the absorbance at 280 nm divided by the extinction coefficient, 1.38. Other protein concentrations were determined by the BCA (bichinchononic acid) procedure (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. Purified mAb were radioiodinated using a chloramine T method to a specific activity of 20-30 μ Ci/ μ g (Greenwood et al., Biochem. J., 89, 114 (1963)). Free 125 I was separated from bound label by passage of the reaction mixture over a Sephadex G-50 column. MAb were conjugated to horseradish peroxidase (HRP) using the method of 25 P. Nakane et al., J. Histochem Cytochem., 22, 1084 (1974). Conjugation ratios generally ranged from 1:1-1:4 mg/mg mAb:HRP.

M. Reciprocal Inhibition Assay

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Radioiodinated mAb was diluted into negative human plasma to produce a tracer solution at 0.2 μ Ci/ml. One hundred microliters of the tracer solution was added to 100 microliters of negative human plasma containing varying concentrations of mAb to be used for competition. A polystyrene bead coated with Dane particles at 2 μ g/ml was added and incubated with this mixture for 2 hrs at 40°C or overnight at room temperature. The bead was washed with distilled water and counted for radioactivity. 35

N. Sandwich Antigen Immunoassays

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Polystyrene beads (6 mm in diameter) were coated with capture antibody at a concentration of 20 μ g/ml for 2 hrs at 40°C. Beads were rinsed with PBS, then incubated with a PBS solution containing 3% BSA for 1 hr at 40°C. Beads were rinsed with PBS containing 3% sucrose, then allowed to air dry.

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Sample (0.2 ml) was incubated overnight at 20-25°C or for 2 hrs at 40°C with the antibody-coated bead. After washing with water, the bead was incubated with 0.2 ml of 125 I labelled- or HRP-conjugated detection antibody for 2 hrs at 40°C. The bead was washed with water and counted for radioactivity in a gamma counter for the radioimmunoassay (RIA), or incubated with a HRP substrate solution (0.3 ml of 0.3% o-phenylenediamine-2-HCl in 0.1 M citrate-phosphate buffer (pH 5.5) containing 0.02% H₂O₂) for the enzyme-linked immunoassay (EIA). The enzymatic reaction was allowed to proceed for 30 minutes at room temperature, then stopped by the addition of 1 ml of 1 N H₂SO₄. Absorbance at 492 nm was measured using a Quantum II spectrophotometer (Abbott Laboratories). 50

O. Results

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1. Purification of Dane Particles

Hepatitis B virions in human plasma were separated in pure form from the bulk of the 22 nm HBsAg

particles and other plasma proteins by sucrose gradient centrifugation. Fractions were monitored by specific immunoassays for the detection of HBcAg, PreS1 Ag, HBsAg, and PreS1 Ag. The peak of S and PreS2 antigen activity co-sedimented with 22 nm particles, whereas PreS1 antigen and HBcAg co-sedimented as a significantly larger particle corresponding to 42 nm Dane particles. These data indicate that PreS1 and HBcAg, but not PreS2 and S could be used to help differentiate Danes from small 22 nm forms. Predominance of L protein (p39, gp41) in Dane particles as compared to purified HBsAg particles was observed by SDS PAGE as has been reported previously by Herrman et al., supra.

10 2. Immunization

Purified Dane particles (subtype ad; similar results can be obtained with ay) were used to immunize mice. Ten of ten Dane-immunized mice produced detectable anti-PreS1 and anti-PreS2 titers as measured by Western blotting and reactivity on the Dane coated bead immunoassay. Some immune response was elicited by inoculation with PreS2 synthetic peptide (120-145). Dane particles also proved to be significantly better immunogens in mice than a synthetic PreS1 peptide (12-53) inoculated as free peptide or coupled to KLH or BSA in adjuvant.

Hybridomas were initially screened by reactivity by solid phase EIA using Dane particles coated onto the solid phase and by Western blotting of Dane particles. Reactive clones were grown in ascites and mAb purified from ascites by protein A affinity chromatography.

3. PreS1 mAb

Purified mAb were characterized by Western blotting reactivity with purified Dane particles of ad and ay subtype, binding to synthetic peptides and recombinant PreS1 or PreS2 proteins, and reciprocal competition studies. Relative affinities were also determined as described in Materials and Methods. A summary of these results is shown in Table 3 for mAb PreS1.

TABLE 3

PHE-51 MONOCLONAL ANTIBODIES

Group	Clone Number	Isotype	Affinity	PHE-51			Western blot reactivity	PHE-51 reactivity
				Inhibition	Reactivity with rPHE-51	Ad		
1	116-9-250	IgG1	10E7-10E9	+	+	+	+	27-35
	116-11-193	IgG2a	10E6-10E7	+	+	+	+	27-35
	116-29-129	IgG1	10E9-10E12	+	+	+	+	27-35
	116-00-179	IgG1	10E9-10E12	+	+	+	+	27-35
2	113-39-104	IgG1	10E6-10E7	+	+	+	+	27-35
	115-16-407	IgG1	10E7-10E9	+	+	+	+	72-102
	116-0-151	IgG1	10E9-10E12	+	+	+	+	72-102
	116-72-270	IgG1	10E7-10E9	+	+	+	+	72-102
3	116-109-364	IgG1	10E7-10E9	+	+	+	+	72-102
	116-192-256	IgG1	10E7-10E9	+	+	+	+	72-102
	116-86-293	IgG1	10E9-10E12	+	+	+	+	72-102
	116-7-100	IgG1	10E7-10E9	+	+	+	+	?
	116-54-142	IgG2a	10E9-10E12	+	+	+	+	?
	116-71-571	IgG1	10E7-10E9	+	+	+	+	?

ND-not done

The grouping of the PreS1 mAbs was determined from reciprocal competition immunoassays in which mAbs competed for binding to denatured Dane particle-coated beads. All PreS1 mAbs could be segregated into three distinct groups from these studies. The group 1 and group 2 PreS1 mAbs bind to rPreS1 and also react with the L protein (p39 and gp42) of Dane particles (subtype ad and ay) by Western blotting. These mAbs do not react with HBsAg protein S (p24 and gp27) or with M proteins (gp33 and gp36). Group 1 and 2 mAbs do not compete for binding to Dane particle-coated beads. Group 1 mAbs specifically bind synthetic peptide (13-51) whereas Group 2 and Group 3 mAbs do not bind this peptide. The Group 1 mAbs also effectively compete with known anti-PreS1 mAb 18/7. Deletion mapping studies indicate that Group 1 mAbs bind to region 27-35 and Group 2 mAbs bind to region 72-102.

The Group 3 anti-PreS1 mAbs behave very differently from the other two groups. These mAbs react only with L protein by Western blotting for ad subtype but show no detectable reactivity for ay subtype. Group 3 did bind in a competeable manner to Dane ad coated beads. These antibodies also did not react with any HBsAg subtypes in a sandwich immunoassay in which AUSZYME II beads were reacted with up to 45 µg/ml of HBsAg, then reacted with ¹²⁵I-labelled mAb. Group 3 mAb showed virtually no binding to rPreS1 either in solid phase immunoassay or by Western blotting. One high affinity anti-PreS2 mAb (50-80-194) showed partial inhibition of the binding of all three groups of PreS1 mAb to Dane particles (21-34%) at high concentration.

All other anti-PreS2 mAb and two anti-S mAb (H35 and H166) exhibited low or negligible inhibition of PreS1 mAb binding. H35 and H166 bind non-competing group-specific "a" determinants and were described previously by Peterson et al., *J. Immunol.*, 132, 920 (1984). The observed partial inhibition results from steric constraints. H166 gave 25% inhibition of Group 1 PreS1 mAb binding but less than 10% for Groups 2 and 3.

4. PreS2 mAb

The PreS2 mAb could be divided into four groups based on reciprocal competition experiments. The profile of reactivity of these mAbs is summarized on Table 4, below.

T A B L E 4

PHE-52 MONOCLONAL ANTIBODIES

Group	Clone Number	Isotype	Affinity	120-145 Peptide Inhibition		rPro-52		Western Blot Reactivity		Endo F Sensitivity % Loss
				Inhibition		rPro-52		Ad	AY	
1	a)	50-00-194	IgG2a	10E9-10E12	+	+	+	gp33/36/39/42	gp33/36/39/42	0
		116-183-406	IgG1	10E7-10E9	+	+	+	gp33/36/39/42	gp33/36/39/42	0
		55-392-260	IgG1	10E6-10E7	+	+	+	gp33/36/39/42	gp33/36/39/42	0
	b)	25-19-117	IgG1	10E9-10E12	+	+	+	gp33/36/39/42	gp33/36/39/42	0
2		116-34-263	IgG1	10E9-10E12	-	-	-	gp33/36	gp33/36	100
		116-94-140	IgG1	10E7-10E9	-	-	-	gp33/36	gp33/36	nd
3		115-32-101	IgG1	10E7-10E9	+/-	-	-	gp33/36	gp33/36	09
		115-145-132	IgG1	10E7-10E9	-	-	-	gp33/36 weak 39/42	gp33/36 weak 39/42	nd

PreS2 group 1 mAb reacted with a linear epitope on both M and L proteins by Western blotting, to a synthetic peptide 120-145, and to a rPreS2 fusion protein containing residues 123-175.

The PreS2 Group 1 mAb may be further subdivided into Groups 1a and 1b. The high affinity Group 1a mAbs (e.g., 50-80-194) completely blocks the binding of the high affinity Group 1b mAb 25-19-117, but 25-19-117 only blocked 50% of 50-80-194 binding to Dane particle-coated beads even at 20 μ g/ml. This differential inhibition probably does not result from differences in mAb affinity for Dane particles since some Group 1a mAbs (e.g., 55-392-260) have significantly lower affinities for Dane particles than 25-19-117. More likely, Group 1a and 1b mAbs bind distinct but closely spaced epitopes so that competition occurs by steric hindrance. This phenomenon is seen between known anti-S mAbs H95 and H35 (D. L. Peterson et al., *J. Immunology*, 132, 920 (1984)).

The Group 2 PreS2 mAbs bind to M protein (gp 33 and gp36) but not S or L (p39 and gp42), indicating that the mAb may bind to a free PreS2 amino terminus or to the carbohydrate moiety at ASN 123. To determine whether a carbohydrate moiety might constitute part of the Group 2 epitope, Dane particles were treated with endoglycosidase F. Gp33 and gp36 were converted to a protein band at 30 kD which corresponds to the deglycosylated M protein (p30). No binding of Group 2 mAbs to p30 was observed indicating that the carbohydrate moiety is an essential part of the binding site. These results are consistent with the lack of reactivity of Group 2 mAbs with the synthetic peptide (120-145) and the rPreS2 produced in *E. coli* which both lack the high mannose carbohydrate chain at ASN 123.

The Group 3 PreS2 mAb gave similar Western blotting patterns to Group 2 in reacting only with M antigen Dane particles. The Group 2 mAb completely blocked the binding of Group 3 mAb to Dane particles, but Group 3 could only partially compete (50%) with Group 2 mAb for binding to Danes. Carbohydrate appears a part of the binding epitope. These data indicate that Groups 2 and 3 mAbs define distinct yet sterically restricted sites.

Group 4 mAb showed no reciprocal inhibition with any other PreS2 mAb groups and reacted strongly to Dane ad subtype but not Dane ay subtype. These mAb were not reactive with rPreS2 or the 120-145 (adw2) peptide. This Mab reacts with PreS2 protein of type aywl.

None of the PreS1 or S mAb inhibited the binding of these PreS2 mAb to Dane particles (ad subtypes). Reciprocally, none of the PreS1 or PreS2 mAb effectively competed with the anti-S mAb H95 and H166.

Example 2. HBV Subtyping

Some clear differences in HBV subtype affinity were observed among mAb groups. These differences are summarized on Table 5, below.

TABLE 5

Subtype	ProS2 Mab				ProS1 Mab			S.M.B.
	1a/1a	1b/1a	1a/2	1a/3	1/1	2/2	2/1	
50-80/116-83	25-19/50-00	50-00/116-34	50-00/115-32	116-00/116-29	116-0/115-16	116-0/116-00	1195/1110	
ayw1	511.00	0.10	1.27	4.21	4.15	201.00	1.02	0.51
ayw2	4.29	1.50	0.03	2.00	4.33	1.70	3.14	0.02
ayw3	4.59	1.39	0.95	3.16	3.59	1.56	1.67	0.01
ayw4	6.43	1.40	1.17	4.14	3.31	1.50	1.23	0.02
ayi	0.50	235.50	0.04	0.75	5.61	106.60	2.20	0.02
adw2	7.20	1.00	1.34	5.22	0.50	60.90	1.30	1414.00
adw4	8.50	6.96	0.06	0.10	4.93	2.05	9.27	95.00
adi	8.50	602.20	0.02	0.04	11.09	1.73	2.09	42.00
adv	4.96	1.42	0.00	3.00	4.50	1.95	1.04	1.34

* Boxed binding ratios are significantly above or below the average ratios for the pair.

PreS1 Group 2 mAb gave nine-fold higher signals in binding experiments with adw4 subtype than PreS1 Group 1 mAb, while its binding to other subtypes was no more than about three times greater. Similarly, PreS2 Group 2 and 3 mAbs gave more than thirty-fold greater reactivity with adr and adw4 subtypes than PreS2 Group 1 mAb, while the Group 1 reactivity was greater than Group 2 or 3 reactivity for the other subtypes. Thus, a PreS2 Group 2 mAb is preferred as a detection antibody in sandwich-type immunoassays, as it is capable of binding substantially equally to all of the HBV subtypes.

Within PreS1 Group 1, one mAb 116-80-179 gave 4-10 fold higher signals for adw2 and adw4 subtypes than other mAbs in this group. One mAb, 115-16-407 in the PreS1 Group 2, gave significantly lower binding to aywl, ayr, and adw2 than other PreS1 mAbs in this group.

In PreS2 Group 1, mAb 50-80-194 reacts 50-fold more strongly with aywl subtype than does 116-183-406. Interestingly, all mAb were generated from mice immunized with Dane particles (ad subtype) except 50-80-194 which was derived from a mouse immunized with SDS-denatured Dane particle subtypes ad and ay and the mice were subsequently pre-fuse boosted with the synthetic peptide 120-145 (adw2 subtype). MAb 25-19-117 was derived from a mouse immunized with synthetic peptide 120-145. MAb 116-34-263 reacts much more strongly with adw4, ayr, and adr subtypes than does 50-80-194.

From these data, it should be possible to determine the HBsAg subtype of various infected individuals. Subtypes d vs. y may be easily distinguished by measuring serum HBsAg reactivity to the known anti-S mAbs, H95 and H10 which react strongly with d or y subtype, respectively. These results indicate that adw4 and adr may be easily differentiated from adw2 and that ayw may be easily distinguished from ayr using PreS2 mAb 50-80-194 and 116-34-263.

Example 3. PreS1 and PreS2 Antigen Assays

A. PreS2 Antigen Assay

Specifically for the PreS2 antigen assays, beads coated with goat anti-HBs (Abbott Laboratories) were used with radio- or enzyme-labelled mAb (50-80-194 or 25-19-117). Most studies were conducted using Auszyme^{RII} beads (Abbott Laboratories) comprising bound polyclonal, guinea pig anti-S antibodies, and a tracer solution containing a mix of ¹²⁵I labelled mAb anti-PreS2 (Group 2: 116-34-263 and/or Group 1: 50-80-194).

B. PreS1 Assay

To detect PreS1 antigen, monoclonal Auszyme^R or Auszyme^{RII} beads were used with radio- or enzyme-labelled mAb 116-29-129 (Group 1). Also, beads coated with mAb 116-80-179 (Group 1) were used with radio- or enzyme-labelled 116-8-151 or 116-72-270 (both Group 2), as the probe.

C. Results

1. PreS2 Antigen Assays

A three-step configuration using goat anti-HBs antibody (Abbott Laboratories) coated beads and PreS2 mAb (25-19-117) (Group 1b) in a second step followed by a detection step using HRP conjugated goat anti-mouse IgG was used for many experiments and gave good sensitivity and selectivity. A two-step procedure with equivalent selectivity and sensitivity has also developed with the Auszyme^{RII} beads and using an HRP conjugated or ¹²⁵I-PreS2 mAb [50-80-194 (Group 1) or 50-80-194 + 116-34-263 (Group 2)] as the detection mAb. One hundred specimens testing negative for HBsAg by the monoclonal Auszyme^R were all unreactive in the PreS2 antigen assays described above. Based on these negative populations, a cutoff for the EIA assay of 0.05 + NCx or 4XNCx for the RIA were chosen (greater than 7 standard deviations from the negative population mean). To quantify PreS2 Ag, dilutions of a hpHBsAg preparation were run in each assay. The percent of total M protein contained in hpHBsAg preparations was determined by scanning densitometry of Coomassie stained SDS gels. The preparation used for these quantitation experiments

contained 15% M protein. Sensitivity of the PreS2 assay was determined to be approximately 1 ng/ml of total hHBsAg or 0.15 ng/ml of M protein.

Recombinant HBsAg containing only the S gene at concentrations of up to 300 µg/ml did not inhibit the anti-PreS2 assay indicating that PreS2 Ag can be detected in the presence of a 80,000-fold excess of S protein. Two hundred plasma specimens from HBV carriers were quantitatively assayed for HBsAg using the monoclonal Auszyme^R assay and for M protein using the PreS2 Ag assays described above. Ninety-nine percent (102/103) of HBeAg positive carriers and 95% (81/85) of anti-HBe positives were reactive by the PreS2 antigen assay. On average, the levels of M proteins are 11-fold higher (and those of HBsAg are 5-fold higher) in the HBeAg positive carriers compared to the anti-HBe positive group.

Specimens with M protein concentrations higher than 10 µg/ml fall exclusively into the HBeAg positive group. There is extensive overlap in M protein concentrations between HBeAg and anti-HBe positive specimens below these values. The HBV DNA concentrations in a subset of these populations was determined and showed poor correlation with M protein (data not shown).

These data taken together suggest that PreS2 antigen does not provide any additional information beyond that obtained from an anti-S specific test (monoclonal Auszyme^R) and does not serve as an accurate indicator of active viral replication.

2. PreS1 Antigen Assay

Three sandwich immunoassays were developed for detection of PreS1 Ag: one used a monoclonal Auszyme^R bead and HRP conjugated PreS1 mAb (116-29-129, Group 1) as detection system; the second used PreS1 mAb (116-80-179, Group 1) as the solid phase capture Ab and HRP conjugated or radioiodinated PreS1 mAb (116-72-270; Group 3) as the detection mAb; and the third utilized Auszyme^R II beads and tracer containing ¹²⁵I labelled 116-29-129 and 116-8-151 (Group 2) as the detection mAb.

To quantify PreS1 Ag, dilutions of purified Dane particles were run on each assay. The percent of total L protein contained in the Dane preparation was determined similarly to the procedure used for M protein. The Dane preparation used for quantitation was about 10% L protein. To determine an assay cutoff, 100 HBsAg negative sera and plasma were run in the assay. All were unreactive when a cutoff of 0.05 + NCx in the EIA or 4 times NCx in the RIA (greater than seven standard deviations from the negative population mean) was used.

All HBeAg positive specimens from HBV carriers (n=229) and 96.6% (57/59) of anti-HBe positive specimens were reactive in the PreS1 Ag assay. PreS1 Ag concentration varied over a wide range from less than 8 ng/ml to greater than 16 µg/ml. No anti-HBe positive specimens had greater than 0.9 µg/ml L antigen compared to 37.5% of the HBeAg positive specimens. Significant overlap in PreS1 Ag concentrations in sera is observed between HBeAg and anti-HBe reactive specimens at L protein concentration in sera less than 0.9 µg/ml.

Ninety-three percent of HBeAg positive specimens had detectable HBV DNA in their sera (mean [HBV-DNA] = 63.8 pg/ml, range 0.5 - 445 pg/ml) compared to 20% of anti-HBe positive specimens (mean [HBV-DNA] = 2.14 pg/ml range 1.7 -2.4 pg/ml).

Although sera with high HBV DNA concentrations tended to have higher concentrations of PreS1 Ag, there was only a moderate quantitative correlation between these markers (R=0.52). A lower quantitative correlation was observed between PreS1 Ag and HBsAg concentrations (R=0.38).

Example 4. Antibody Assays

To perform an assay for anti-PreS2 antibodies in the blood of seropositive individuals, 200 µl of serum was added to a single 0.25 inch polystyrene bead coated with HBsAg comprising reduced and iodoacetamide-capped S protein. The mixture was incubated for 18 hrs at 25° C; then washed with water and 200 µl of ¹²⁵I-labelled mAb 50-80-194 added (1 µC/ml) in a diluent containing animal sera. The mixture was incubated for 2.0 hrs at 40° C, washed to remove free mAb, and the bead was counted. The time course HBsAg, anti-HBs antibody and anti-PreS2 antibody for one HBV patient is summarized on Table 6, below.

Table 6

Patients's Blood Sampled on Day	Auszyme ^R HBsAg	Ausab ^R (anti-HBs)	Competitive Anti-PreS Ab	
			S/CO*	Interpretation
1	-	-	1.787	-
31	+	-	1.782	-
36	+	-	1.721	-
44	-	+	1.065	+
52	-	+	1.080	+
62	-	+	0.944	+
93	-	+	0.211	+
122	-	+	0.183	++

* Ratio of cpm (sample)/cpm(cut-off); negative control value = 2; if S/CO < 1.1, then sample is considered positive (+) for antibody against PreS2 protein.

Discussion

MAB were generated which defined at least three distinct epitopes in the PreS1 region and four distinct epitopes within the PreS2 region of the HBV envelope. As observed by Western blotting, all of these patterns are retained after treatment of Dane particles by heat, SDS, and B-mercaptoethanol (BME). These treatments destroy most of the antigenic sites on the S protein. Determinants may be linear epitopes or are conformational ones which can reform after transfer of protein to nitrocellulose. No cysteines are present in PreS1 and PreS2 regions so that proper reforming of disulfide bonds is not necessary to retain the native conformation. This is in distinct contrast to most antigenic epitopes of S (p24 and gp27) which are highly dependent on disulfide bond formation and proper assembly into particles. Most of the S determinants are destroyed during the SDS/BME denaturation step during sample preparation for Western blotting.

Because Western blotting was a key method for screening anti-PreS2 and anti-PreS1 mAb, it is possible that mAbs binding to conformation-dependent epitopes in these regions may be missed. All mAb were also tested for reactivity with native Dane particles and, therefore, all epitopes defined by these mAbs must be exposed on the viral surface.

The data demonstrate that for one group of anti-PreS2 mAb (Group 2), the glycan at asparagine (Asn) 123 in the PreS2 region comprises an essential part of the determinant. Because this mAb does not bind to other human serum glycans, it is likely that the antigenic determinant consists of both carbohydrate and protein as has been demonstrated for the human blood group protein, glycophorin A (MN antigen).

The present PreS2 and PreS1 mAbs have proven useful in developing specific and sensitive immunoassays to detect M and L protein in human sera and plasma. Previous reports claimed an absolute correlation between the presence of serum markers for HBeAg and the presence of M protein (M. Imai et al., *Gastroent.*, 76, 242 (1979)). Another claim in the literature was that the presence or absence of PreS1 Ag correlates with infectivity and the presence of HBV DNA. The data support the work of Hu et al. (cite), which showed a lack of qualitative correlation with the HBeAg status, although HBeAg individuals have a higher average concentration of HBsAg, PreS2 Ag, PreS1 Ag and HBV DNA.

Virtually all HBsAg carriers were found to have detectable PreS1 and PreS2 Ag in their sera or plasma when the present, sensitive immunoassay is used for detection. It is clear then that this PreS Ag assay gives similar diagnostic and screening information to that from an S protein (HBsAg) specific test (monoclonal Auszyme^R). Quantitation of HBsAg, PreS1, and PreS2 antigen does give some additional information, in that individuals with S, M, and L protein concentrations above the threshold values of 100 µg/ml, 10 µg/ml and 0.8 µg/ml, respectively, were HBeAg positive and HBV DNA positive (sens=0.5 pg/ml). However, for the majority of HBV-infected individuals with hepatitis B envelope antigen concentrations below these threshold values, anti-HBe/HBeAg status or HBV DNA detectability could not be accurately predicted.

Other uses for high affinity PreS2 and PreS1 mAbs include mapping the hepatocyte binding region of the L protein, as well as intracellular and cell surface staining of HBV infected hepatocytes and experimen-

tally transfected hepatoma cell lines. Lower-affinity PreS1 and PreS2 mAbs of the invention have been successfully used to affinity purify recombinant and plasma derived PreS2 and PreS1 containing proteins at high yields.

Samples of certain of the hybridoma lines producing monoclonal antibodies of the invention have been deposited in accord with the Budapest Treaty in the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 and have received the designations shown on Table 7, below.

Table 7

Hybridoma	ATCC Designation
HBV 116-29-129	HB 10118
HBV 116-8-151	HB 10119
HBV 116-86-293	HB 10120
HBV 50-80-194	HB 10121
HBV 116-34-263	HB 10122

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

Claims

1. A monoclonal antibody which binds to an epitope in the region of the hepatitis B virus (HBV) PreS1 protein comprising the amino acid sequence:
HQLDPAFGANS

and wherein said monoclonal antibody does not bind to the HBV M protein.

2. A monoclonal antibody which binds to an epitope in the region of the hepatitis B virus (HBV) PreS1 protein comprising the amino acid sequence:
HGGLLGWSPQAQGIMQTL PANPPASTNRQSG

and wherein said monoclonal antibody does not bind to the HBV M protein.

3. A monoclonal antibody which binds to an epitope in the region of the hepatitis B virus (HBV) PreS2 protein comprising the amino acid sequence:
MQWNSTAFHQTLQDPRVRGLYPAGG

and wherein said monoclonal antibody binds to the HBV M protein and the HBV L protein.

4. A monoclonal antibody which binds to an epitope comprising the carbohydrate moiety at asparagine (123) of the hepatitis B (HBV) PreS2 protein, wherein said monoclonal antibody binds to the HBV M protein but not to the HBV S protein or the HBV L protein.

5. The monoclonal antibody of claims 1, 2, 3, or 4 which further comprises a detectable amount of a bound label comprising a radioisotope or an enzyme.

6. A method to detect hepatitis B virus (HBV) PreS1 protein in a sample of a physiological material infected with HBV comprising:

(a) reacting said physiological material with an immobilized antibody which binds to HBV protein comprising the PreS1 protein, to form a complex between said first antibody and said PreS1-containing protein;

(b) reacting said complex with the monoclonal antibody (mAb) of claim 1, the mAb of claim 2, or a mixture thereof; wherein said mAb comprises a detectable label, to yield a ternary complex; and

(c) detecting said label so as to measure the concentration of HBV PreS1 protein in said material, wherein the presence of HBV protein does not interfere with the measurement of the PreS1 concentration.

7. A method to detect hepatitis B virus (HBV) PreS2 protein in a sample of a physiological material infected with HBV comprising:

(a) reacting said physiological material with an immobilized antibody which binds to HBV protein comprising the PreS2 protein, to form a complex between said first antibody and said PreS1-containing protein;

(b) reacting said complex with the monoclonal antibody (mAb) of claim 3, the mAb of claim 4, or a mixture thereof; wherein said mAb comprises a detectable label, to yield a ternary complex; and

(c) detecting said label so as to measure the concentration of HBV PreS2 protein in said material, wherein the presence of HBV protein S does not interfere with the measurement of the PreS2 concentration.

5 8. A method to completely subtype a hepatitis B virus which comprises:

(a) reacting a first portion of a sample of an immobilized hepatitis B surface antigen of known ay or ad subtype with a first monoclonal antibody which binds to HBV PreS1 protein or PreS2 protein;

10 (b) reacting a second portion of said sample with a second monoclonal antibody which binds to PreS1 protein or a PreS2 protein, wherein said first monoclonal antibody and said second monoclonal antibody each comprise a detectable label; and

(c) calculating the ratio of bound labelled first monoclonal antibody:bound labelled second monoclonal antibody; wherein said ratio is indicative of the complete subtype of said sample.

15 9. A method for raising plasma immunogenecity to hepatitis B virus (HBV) comprising administering to an individual a pharmaceutical unit dosage form comprising an effective amount of at least one of the monoclonal antibodies of claims 1, 2, 3, or 4.

10. A method for measuring the amount of antibodies against PreS protein in a sample of physiological material infected with HBV comprising:

(a) combining preselected amounts of

20 (i) an immobilized antigen comprising PreS protein,

(ii) a physiological material which comprises an antibody which binds to said PreS protein, and

(iii) a monoclonal antibody which binds to said PreS protein, wherein said monoclonal antibody comprises a detectable label; so that said monoclonal antibody and said antibody in said physiological material bind competitively to said immobilized antigen; and (b) measuring the amount of label which is bound to said immobilized antigen.

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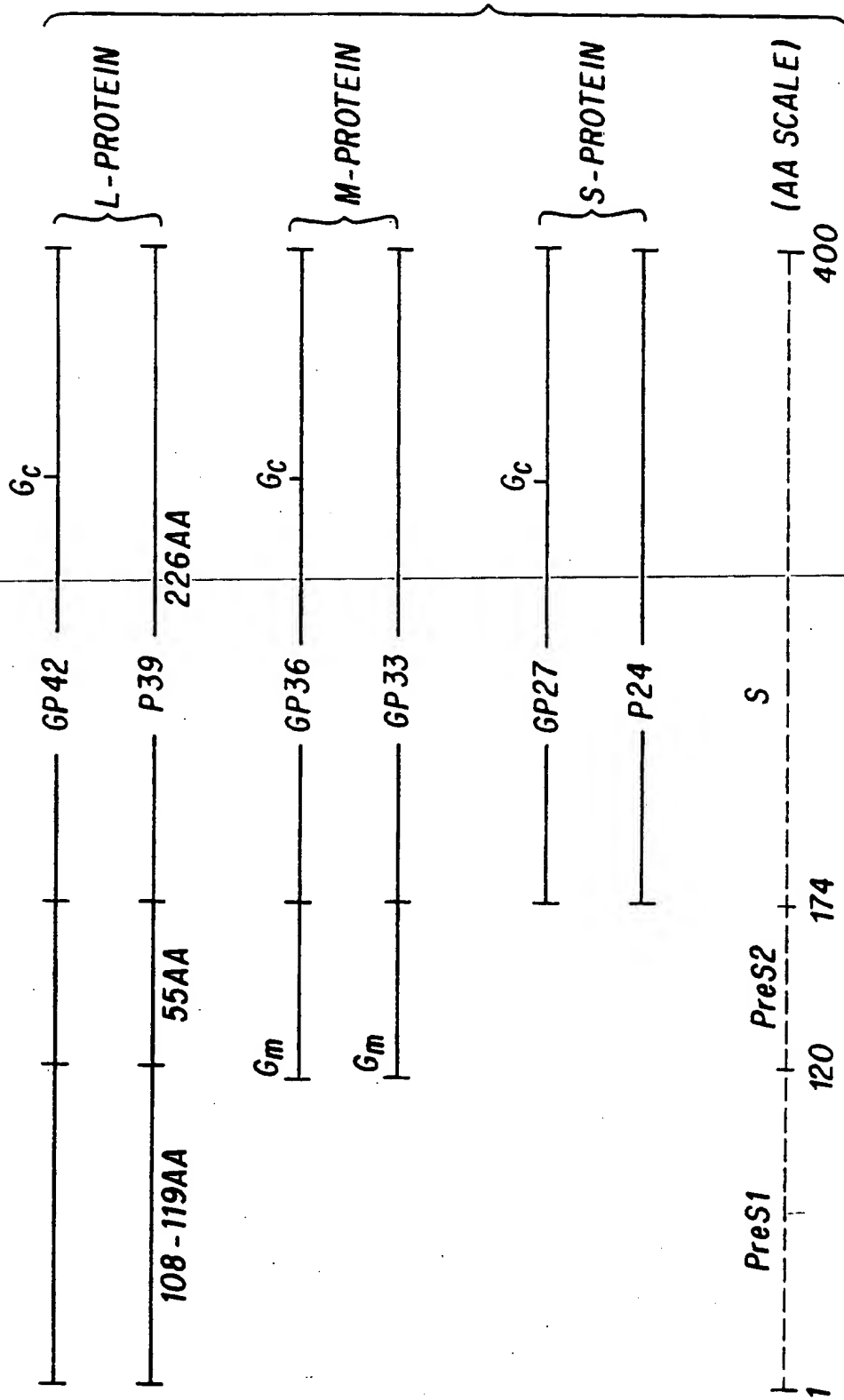


FIG. 1



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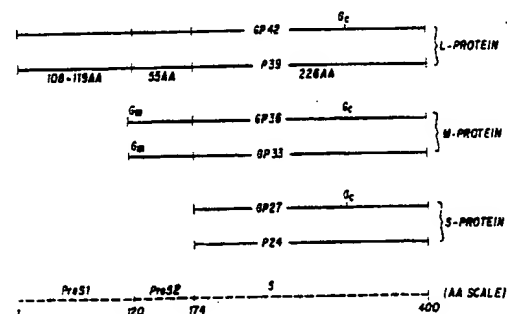
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Monoclonal antibodies to preS2 and preS1 polypeptides of the hepatitis B viral envelope.

Murine monoclonal antibodies are provided which bind specifically to the PreS1 and PreS2 polypeptides of the hepatitis B virus surface antigen (HBsAg) complex and which are useful to assay for PreS1 or PreS2 in samples of physiological material. The antibodies can also be used to subtype HBV, to raise the immunogenicity of blood plasma against HBV, to measure anti-PreS1 and anti-PreS2 antibodies, and to identify and characterize antigenic epitopes of PreS1 and PreS2 which may be useful in HBV subunit vaccines.



EP 0 389 983 A3



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PARTIAL EUROPEAN SEARCH REPORT
which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number

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INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely: 1-8, 10 Claims searched incompletely: 9 Claims not searched: Reason for the limitation of the search: Article 52(4) EPC</p>			
Place of search VIENNA		Date of completion of the search 29-10-1990	Examiner WOLF
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT
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